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14. ABSTRACT The goal of this project is to develop miR-326 small molecule inhibitors (SMIs) for the treatment of Multiple Sclerosis (MS). Our global approach consists of creating stable transfectants with a luciferase reporter gene and 3' target sequence for miR-326. The addition of hsa-miR-326 (delivered either by lentivirus or cotransfection) should result in reduced fluorescence and the addition of putative inhibitors will restore the luciferase-dependent fluorescence. A great deal of time on this project has been devoted to troubleshooting unforeseen problems with transfection and establishing stable cell lines expressing constructs of interest. During the supported period we successfully generated plasmids to be used for stable and transient transfections of miR-326 and miR-21 (as control miRNA).					
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INTRODUCTION

The goal of this project is to develop miR-326 small molecule inhibitors (SMIs) for the treatment of Multiple Sclerosis (MS).

BODY

MicroRNAs are small non-coding RNAs that function as gene regulators by interfering with translation or stability of target mRNAs. They have been shown to exert profound effects in a variety of disease models; they act as tumor suppressors cancer and influence inflammation. One microRNA, miR-326, affects development of Th17 cells, a pathogenic T cell subset associated with multiple sclerosis. Moreover, this microRNA is elevated in the brains of MS patients. These observations suggest that small molecule inhibitors of miR-326 could be used therapeutically to limit inflammation associated with MS and reduce disease severity. The goal of this project is to develop miR-326 small molecule inhibitors (SMIs) for the treatment of Multiple Sclerosis (MS). Our global approach consists of creating stable transfectants with a luciferase reporter gene and 3' target sequence for miR-326. The addition of hsa-miR-326 (delivered either by lentivirus or cotransfection) should result in reduced fluorescence and the addition of putative inhibitors will restore the luciferase-dependent fluorescence. A great deal of time on this project has been devoted to troubleshooting unforeseen problems with transfection and establishing stable cell lines expressing constructs of interest.

Most cell lines express many miRNAs. In contrast, HeLa cells are unusual in that they express only miR-21. Our goal is to take advantage of this by generating HeLa transfectants that also express miR-326. By comparing HeLa cells with or without miR-326, this system provides an ideal control for screening for SMIs that are miR-326 specific or can interfere with other miRs. As a control, we transfected either pmirGLO Dual-Luciferase miRNA Target Expression Vector with no miRNA binding site (empty vector) or pmirGLO containing the miR-21 binding sequence. As expected, there was robust luciferase activity with the empty vector, while there was little activity using the pmirGLO-miR21 plasmid because the endogenous miR-21 was able to bind the luciferase mRNA and suppress activity (Figure 1).

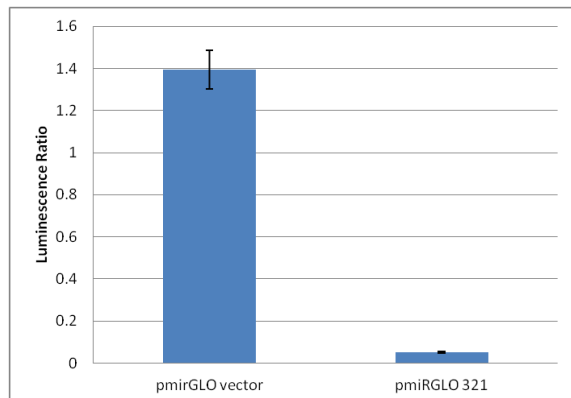


Figure 1. HeLa cells transfected with miR21 binding sequence

HeLa cells (7000 cells/well) were transfected with 200ng of pmirGLO empty vector or pmirGLO 21 (containing mir-21 binding sequence) using Lipofectamine. After 48 hours Dual Glo substrate was added to the cells and luciferase activity and Renilla Luciferase activity were measured. Results are calculated as ratio of firefly:Renilla luminescence and normalized to the ratio from control wells.

Because MCF-7 cells are reported to express miR-326 as well as miR-21, we employed these cells as an additional control. Again, the pmirGLO-mi21 vector had little luciferase activity compared with empty vector. However we did not detect a loss of luciferase activity when

pmirGLO-mir326 was transfected, suggesting that the amount of endogenous miR-326 was not sufficient to inhibit translation of the reporter construct. To overcome this, we co-transfected the pmirGLO plasmids with pMR04, which encodes miR-326. There was no detectable reduction in luciferase activity (Figure 2). Sequencing of the pmirGLO-mi326 plasmid showed that the

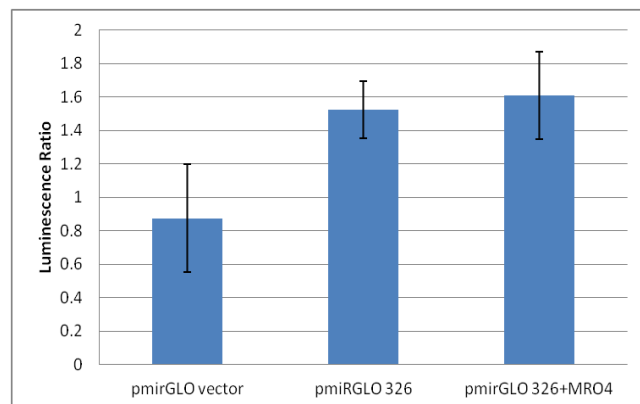


Figure 2. MCF-7 cell line transfected with miR326 binding sequence and miR326

MCF-7 cells (20000 cells/well) were transfected with 200ng of pmirGLO empty vector, pmirGLO 326 (containing mir-326 binding sequence), or pmirGLO326 and pMR04 (encoding mir-326) using Lipofectamine . After 48 hours Dual Glo substrate was added to the cells and Firefly and Renilla Luciferase activities were measured. Results are calculated as ratio of firefly:Renilla luminescence and normalized to the ratio from control wells.

construct had the appropriate binding site.

Since the pMR04 plasmid also encodes GFP,

we could determine the frequency of cells that were transfected with that plasmid by using flow cytometry. Very few cells expressed the GFP plasmid, and by extension, little miR-326.

Since co-transfections with the MR04 plasmid were not successful, we then co-transfected the cells with a miR-326 mimic. This is a synthetic RNA encoding the precursor form of miR-326. Again, there was no evidence that the mimic was able to suppress luciferase activity. We are currently in the process of creating stably transfected cell lines that express miR-326. The pMR04 plasmid encodes a puromycin selection marker to facilitate establishment of stable cell lines. Since puromycin has a very sharp kill curve, we are currently identifying an optimal amount of puromycin that will not kill transfected cells.

Our initial results were obtained with the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega), which has luciferase linked to the miR-326 binding site.

KEY RESEARCH ACCOMPLISHMENTS

- pmirGLO Dual-Luciferase miRNA Target Expression Vector: used as control. (Since there is no insert, the luciferase signal should be high).
- pmirGLO 326 – has the insert for the miR-326 binding site. (If miR-326 is expressed, luciferase should be reduced).
- pmirGLO 21 – has the insert for the miR-21 binding site, to be used as control miRNA during compound screening. (If inhibitor affects miR-21, luciferase should be increased).

REPORTABLE OUTCOMES

We developed plasmids that contain specific inserts as described in Key Research Accomplishments

CONCLUSION

To introduce miR-326 into the cells, we selected the plasmid pEZX-MR04 vector (GeneCopoeia) encoding the precursor miRNA for human miR-326. If expression of miR-326 remains problematic, we are prepared to use lentiviral based vectors that are more resistant to epigenetic silencing. Once the stable transfectants are generated, we will perform transient transfections with the pmirGlo vectors to ensure that luciferase is reduced in the presence of miR-326. This system can then be adapted to screen for the small molecule inhibitors of miR-326.

REFERENCES

None

APPENDICES

None

SUPPORTING DATA

None